

RESEARCH ARTICLE

HeLa Cells Containing a Truncated Form of DNA Polymerase Beta are More Sensitized to Alkylating Agents than to Agents Inducing Oxidative Stress

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Abstract

The present study was aimed at determining the effects of alkylating and oxidative stress inducing agents on a newly identified variant of DNA polymerase beta (pol β Δ 208–304) specific for ovarian cancer. Pol β Δ 208–304 has a deletion of exons 11–13 which lie in the catalytic part of enzyme. We compared the effect of these chemicals on HeLa cells and HeLa cells stably transfected with this variant cloned into in pcDNA1/neo vector by MTT, colony forming and apoptosis assays. Pol β Δ ₂₀₈₋₃₀₄ cells exhibited greater sensitivity to an alkylating agent and less sensitivity towards H₂O₂ and UV when compared with HeLa cells alone. It has been shown that cell death in Pol β Δ ₂₀₈₋₃₀₄ transfected HeLa cells is mediated by the caspase 9 cascade. Exon 11 has nucleotidyl selection activity, while exons 12 and 13 have dNTP selection activity. Hence deletion of this part may affect polymerizing activity although single strand binding and double strand binding activity may remain same. The lack of this part may adversely affect catalytic activity of DNA polymerase beta so that the variant may act as a dominant negative mutant. This would represent clinical significance if translated into a clinical setting because resistance to radiation or chemotherapy during the relapse of the disease could be potentially overcome by this approach.

Keywords: Mutation - DNA polymerase beta - base excision repair - apoptosis - caspase

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Introduction

Human body is being exposed to different kinds of carcinogens, radiation every day. These constant bombardments are causing the DNA damage both in the nucleus and in the mitochondria. In order to manage these high frequencies of DNA damage, nature has developed different DNA repair mechanisms that can be run in parallel to repair the DNA. Base Excision Repair (BER) pathway is one of these pathways, that highly involves the repair of alkylating agent mediated DNA damage by single nucleotide repair pathway (short-patch repair pathway) and the other 2–8 nt repair pathway or long patch repair pathway (Sobol et al., 1996; Fortini et al., 2003; Slupphaug et al., 2003).

The loss of BER activity due to the inefficient function of any of these BER proteins may lead to or further contributes to the development of cancer, neurodegenerative disorders; etc (Singhal et al., 1995; Wilson et al., 2000; Idris et al., 2002). In order to repair efficiently, the activities of BER proteins are tightly regulated. DNA polymerase β is the smallest enzyme in BER pathway. It belongs to the X family of polymerase (Wilson et al., 2000). It is a single copy 34kb gene consisting of 14 exons (Sobol et al., 1996). It is located

in chromosome no 8p12 (Beard and Wilson, 2006). DNA Pol β encodes a 39 kDa protein consisting of 335 amino acids with two subunits, small subunit of 8 kDa (1 amino acids residues to 87 amino acids) and large subunit of 31 kDa (88 amino acids residues to 335 amino acids) connected by a protease sensitive hinge region (Beard & Wilson, 2000). The catalytic property of DNA polymerase β resides in the Carboxyl terminal region of the 31 kDa domain, whereas the amino terminal 8 kDa domain shows a single strand DNA binding activity. This small subunit have single strand DNA binding activity and dRP lyase activity encoded by exons I–IV, its activity is necessary to direct Pol β to short gaps possessing a 5'-phosphate termini (Wilson et al., 2000; Sobol and Wilson, 2001). The large subunit has double strand DNA binding activity and catalytic activity.

DNA polymerase beta is linked to cancer which was detected in different cell lines and tumor tissue sample of cancer (Bhattacharyya et al., 1999a; Bhattacharyya et al., 1999b; Chen et al., 2000; Bhattacharyya et al., 2001; Chen et al., 2002; Khanra et al., 2012a, 2012b, 2012d). It was claimed that 30% cases of cancer polymerase beta have mutation (Starcevic et al., 2004; Lang et al., 2007). In gastric cancer patients out of 20 samples 6 (30%) have pol beta mutation (Iwanaga et al., 1999). In colorectal cancer

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6 in 8 patients (75%) have polymerase beta mutation (Lang et al., 2004). In breast cancer, 26%, esophageal cancer, 40% have mutation (Dong et al., 2002). Almost 36% tumors express polymerase beta variant in which deletion of exon 11 is highly common. This variant of polymerase beta is expressed in colorectal, breast, lungs adenocarcinomas (Sweasy et al., 2005). In colorectal cancer K289M (Sweasy et al., 2005) was detected which has less BER activity. Expression of cancer-associated pol β variants in mouse cells could lead to a series of cancer associated phenotypes, including an increased mutation frequency and the induction of cellular transformation (Sweasy et al., 2005). The gastric cancer associated variant E295K has a phenotype that could be related with etiology of cancer (Lang et al., 2007). This variant is unable to catalyze DNA synthesis but have DNA binding and dRP lyase activity same as wild type (WT) pol β (Lang et al., 2007).

Lang et al (2007) experimentally showed that this mutant when expressed in MEFs, conferred a dominant-negative phenotype and sensitivity to MMS, induced more sister chromatid exchanges (SECs) per nucleus than Wild type (WT) and it causes cellular transformation that could lead to tumorigenesis or tumor progression (Lang et al., 2004). Another Gastric cancer associated variant Leu22Pro (L22P) exhibits little dRP lyase activity but retain its polymerase activity. In 22 residues of DNA pol β has no direct contact with the DNA, L22P variant has reduced DNA binding affinity. The L22P variant protein is deficient in base excision repair (Dalal et al., 2008). In another Gastric cancer variant (cys239arg), cys 239 is located in inflexible loop region that when altered reduced the accuracy of DNA synthesis Dobashi et al., 1994. In prostate cancer, I260M variant is located in hinge region of polymerase beta (Dalal et al., 2005). Ile260 is a key residue of the hydrophobic hinge. This hinge influences the geometry of the DNA within the polymerase active site that is important for accurate DNA synthesis and important for the closing of the polymerase and important for conformation change after substrate binding and fidelity of protein, results inaccurate DNA synthesis when replaced by Methionine. Characterization of the I260M variant shows that it has a functional phenotype that could be linked to the etiology or malignant progression of human cancer (Dalal et al., 2005). Another DNA pol β variant D246V has been reported which is less efficient than WT for incorporation of AZT in a recessed primer template (Kosa et al., 1999). This Asp residues at position 246 helps to maintain the proper positioning of the DNA within the active site of DNA pol β . Valine at the tip of the loop results in mis-incorporation resulting from an altered or misaligned DNA structure within the active site (Kosa et al., 1999; Dalal et al., 2004). Bhattacharyya et al (1999) reported three variant of pol β in squamous, non-small or large cell carcinomas. The common variant was large deletion of 87 bp of exon 11 which lies in catalytic part of polymerase beta. Another variant of 140bp deletion along with addition of exon alpha was detected. Functional study of 87 bp deleted DNA pol β shows that it acts as dominant negative manner. This variant is unable to bind properly to double strand DNA and has lost repair activity

(Bhattacharyya et al., 1997; Bhattacharyya et al., 2000) thus acts as a dominant negative manner. In addition, when this variant is expressed in Mouse Embryonic fibroblast (MEFs) cell line, which induced tumor occurrence in nude mice (Sweasy et al., 2005). So above evidence indicate that Pol β variants are linked to etiology of cancer.

Genomic stability is disrupted by environmental carcinogen as well as metabolically produced oxidative stress, metabolic by-product, errors during DNA replication and DNA recombination contribute to the mutations. It is estimated that BER repairs 10 4 lesions per cell per day and this proofreading is vital to avoid genomic instability (Lindahl, 1993; Yamtich and Sweasy, 2010). By and large, under normal circumstances, this immense error burden is successfully dealt with by the highly coordinated cellular DNA repair mechanisms thus maintaining genomic integrity.

Alkylating agents are mono-functional or bi-functional (two reactive groups) carcinogen that are able to form intra-chain and inter-chain cross-links on DNA directly. Nucleotides can be alkylated at several different positions in nitrogen and oxygen and it's biological consequences are diverse (Friedbarg EC 2003). MMS, NMU are mono alkylating agent directly incorporated in DNA. MMS is an SN2 alkylating agent that interacts with nucleophilic centers forms 7-methylguanine and 3'-methyladenine adducts in DNA (Hoffman & Hecht 1985; Gu et al., 2010). NMU add methyl group to DNA. Endogenous alkylating agent O6-meG interferes base pairing and cause G:C to A:T transition mutations. Other endogenous alkylating agents 7-methylguanines (7meG) are unstable and spontaneously degrade to AP sites create single strand break (Ochs et al., 1999; Oshima et al., 2003). It was detected that Pol β knockout cell lines are highly sensitive to methylating agents.

A UV (100-400 nm) ray is a strong mutagenic agent which creates various lesions. UV A (320-400 nm) is mutagenic consists mostly of single strand breaks in DNA (Mouret et al., 2006; Kong et al., 2009). UVB (295-320 nm) is involved in direct formation of thymine dimer or other pyrimidine dimer double strand DNA breaks. UV-C (100-295 nm), can generate hydroxyl (\bullet OH) and oxygen radicals(\bullet O₂), \bullet OH mainly attacks DNA integrity by removing hydrogen from the deoxyribose backbone or addition of double bonds to DNA bases or resulting base modification including multitude of altered bases, base loss, and strand breaks. The most common lesions created by UV are cyclobutane pyrimidine dimer (CPDs) and Pyrimidine-pyrimidine (6-4) photoproducts (6,4-PP) at the adjacent pyrimidine. The formation of a pyrimidine dimer causes a bend in the DNA helix and DNA polymerase cannot read the DNA template as a result DNA replication is prevented by these pyrimidine dimer, which leads to mutation. The formation of a pyrimidione (6-4) photoproduct mimics an abasic site (Douki et al., 2003).

In our lab we have reported some point mutations in the genomic DNA of ovarian tumor samples (Khanra et al., 2012a,b,c). These mutations were detected at exon intron junction region of exon 8, exon 9 and exon 11 by SSCP analysis (Khanra et al., 2012c). We also have detected cDNA mutation in these samples by RT-PCR

and Western blot analysis (Khanra et al., 2012d). One of the cDNA mutations have deletion of exon 11-13 which lies in catalytic part of DNA polymerase beta. Exon 11 consists of 29 amino acids in the region of 208-236, Exon 12 consists of 21 amino acids in the region of 237- 258, exon 13 is 45 amino acids which resides within amino acid residues 259-304.

In this report, the functional consequence of the loss of three exons of pol β in response to alkylating agents such as Methyl methanesulfonate (MMS) and N-methyl-N-nitrosourea (NMU) and oxidative stress inducing agents such as UV & H₂O₂ have been studied. The present variant of pol β detected in ovarian cancer (Khanra et al., 2012d) has lost exon 11-13 that catalytic part is responsible for dNTP selection, nucleotidyl transferase activity and it would be interesting to determine the function of this mutant in response to alkylating agent, UV and oxidative agents.

Materials and Methods

Cloning of truncated form of DNA polymerase beta in pCDNAIneo vector

Pol β Δ ₂₀₈₋₃₀₄ cDNA was amplified from tumor RNA sample using primers of full length pol β cDNA (Khanra et al., 2012d) and cloned in a vector pCDNAIneo (Invitrogen). The orientation and sequence of the mutated product was confirmed by sequencing before transecting vector pCDNAI/neo in HeLa cell line.

Transfection of Pol β Δ ₂₀₈₋₃₀₄ in HeLa cell line and stable cell line preparation

HeLa cells were cultured in DMEM medium with 10% fetal calf serum (Gibco; USA) and 100 U/mL penicillin and 100U/mL streptomycin at 37°C in a humidified atmosphere with 5% CO₂ in an incubator (SANYO, Japan). Cells were transfected by calcium phosphate co-precipitation method and Transfected cells were selected in G418 containing medium 100 μ g/ μ l.

RT-PCR analysis for expression of truncated form of Pol β

RNAs were isolated from Pol β Δ ₂₀₈₋₃₀₄ transfected cell line, HeLa cell line and only vector transfected cell line without Pol β Δ ₂₀₈₋₃₀₄ and RT-PCR was done to detect the expression of Pol β Δ ₂₀₈₋₃₀₄ in transcriptional level. As per manufacturers' instruction RNAs were isolated using RNA isolation kit (Trisure, Biotline). To eliminate the possibility of genomic DNA contamination, reaction was also set up without reversed transcriptase enzyme

Western blot analysis for detecting expression of truncated DNA polymerase beta

After preparing stable cell line, cell lysates were made and western blot (Bhattacharyya et al., 2001) was done to confirm transfection of Pol β Δ ₂₀₈₋₃₀₄ in cell line. Total cell lysates were isolated from transfected cell lines and control cell lines by using lysis buffer containing 10mM Tris-HCl (PH 8), 1mM EDTA, 400mM NaCl, 2mM beta mercaptoethanol, freshly prepared 1mM PMSF, and protease inhibitors (Roche applied Science). Proteins were denatured in 900 C with SDS loading dye separated on

12.5 % SDS-PAGE gel. Detection of protein was done by using anti-Pol β monoclonal primary antibody (Novus) by diluting 1:5000 in Phosphate-Buffered Saline (PBS) containing 0.05% Tween-20 and 5% nonfat, dry milk. Incubation with secondary antibodies (SC-2020; 1:10,000) and detection reaction were performed according to manufacturer's instruction (PIERCE).

Study of Cytotoxicity

2.5.1. Alkylating agent (MMS, NMU) and UV sensitivity assay by MTT: HeLa cell and Pol β Δ ₂₀₈₋₃₀₄ transfected cells are plated in 96 well plates. After 12-24 hrs cell medium was discarded. Cells were treated with alkylating agent (NMU, MMS) of different concentrations. Then after 24hrs 5mg/ml concentration of MTT (dissolved in PBS) were added in each well. After 4 hours MTT was removed from each well and 100 μ l DMSO was added in each well. Absorbency was measured in 540 nm (Hermann et al., 1994; Hirsh et al., 2009). The sensitivity of Pol β Δ ₂₀₈₋₃₀₄ in chemical treatment was detected by calculating rate of survival using following formula: inhibition rate (%) = (1 – OD A1/A0) x100. Here A1 is absorbency of treated cells, and A0 is absorbency of control cell without treatment. Cell plated in above mentioned protocol and treated after 24 hrs with MMS different doses in Serum free medium. All the assays were done in triplicate.

H₂O₂, UV sensitivity detection by MTT assay

Cell survivability was analyzed using a 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay (Sigma, MO, USA) after treatment with H₂O₂, UV (Krahn et al., 2004). HeLa cells and Pol β Δ ₂₀₈₋₃₀₄ transfected cells were plated onto 96 well plates (1500/well) and after 24hrs medium was discarded and serum free medium was added before 1 hr of treatment. H₂O₂ of different concentrations (12 μ M -1000 μ M) was added, medium was discarded after 1 hour and new medium added after 12 and half hours cells treated with 10 μ l MTT dissolved in PBS. After 3 and half hours, MTT was removed from each well and 100 μ l DMSO was added to each well.

For UV radiation experiment, cells were exposed to different doses of UV light (Stratagene UV-C bulbs, 254 nm) at a dose in between 5-50 J/m². Then 100 μ l of serum free medium was added to each well. After 22 hours of treatment, 10 μ l MTT (dissolved in PBS) was added with a final concentration of 0.5mg /ml. After 4 hours, medium was removed from each well and 100 μ l DMSO was added to each well. Absorbency measured at 540 nM. All the assays were done in triplicate.

Colony forming assay

Exponentially grown cells were seeded in 35 mm dishes (300/ml) and next day treated with UV at 5-15 J/ m², MMS of 0.125-2 μ M, NMU of 1-100 μ M, H₂O₂ of 0.05 to 2 μ M concentrations. After treatment, the cells were washed with PBS and fresh medium was added and cultured in complete medium for 2 weeks. After 14 days of incubation, colonies were fixed with ethanol, stained with Giemsa (25% in ethanol), counted and calculated

as percent of colony following this formula: Untreated/controls x100. All the assays were done in triplicate.

Study of apoptosis

Sensitivity study by DNA fragmentation assay against MMS, NMU, H_2O_2 , and UV: DNA Pol $\beta\Delta_{208-304}$ cells (5×10^5 cells) are seeded in 100mm dish. To assess the fragmentation of cellular DNA into the characteristic apoptotic ladder, cells were treated with the mutagen (UV, NMU, MMS, and H_2O_2) of different time (6, 9, 12 h) post treatment incubation. Then cells were harvested by trypsinization and lysed in ice-cold cell lysis buffer (10 mM Tris, pH 7.5; 1 mM EDTA; 0.5% Triton X-100). Then lysed cells were incubated with 0.1 % SDS and 300 mg/ml of Proteinase K at 56°C overnight. Aqueous phases were extracted with phenol/chloroform and the DNA samples were precipitated with cold ethanol in the presence of $\frac{1}{2}$ volume of 10M ammonium acetate, dried and dissolved by heating at 55°C in TE buffer before loading in a gel. DNA subjected to electrophoresis using 1.8 % agarose in Tris-borate buffer. DNA was visualized by staining with ethidium bromide and photographed in gel.

Caspase activation detection

For caspase 3, 9 activation assays, whole cell extracts were made from cells treated with UV and H_2O_2 with either for different time period or concentrations. Cell lysates were prepared by using lysis buffer containing 20mM Tris-HCl pH 8, 150mM NaCl, 1mM of EDTA, 1mM EGTA and Protease inhibitor (Protease inhibitor mini kit; Roche applied Bioscience). Proteins were electrophoretically separated on 12.5% gel SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was pre-incubated in TBST with 5% blocker (Fat free milk) for 30 mins. Then washed with TBST for 5 mins and incubated in primary antibody of caspase 3, caspase 9 (Cell signaling cat#9668, #9505; dilution 1:1000) for overnight as per manufacturer's instruction. Membrane was rinsed with TBST for 1 hr by changing TBST 4-5 times and incubated afterwards with HRP conjugated secondary antibody with 2.5 % blocker (Bio-Rad; cat#170-6516, Caspase3-Goat anti-mouse IgG; 1:1000; Cell signaling, cat #7074; HRP conjugate anti rabbit antibody 1:1000 conc.) for 1hr. After washing with TBST, bands were visualized by using Super Signal west pico (Pierce, IL).

Statistical analysis

Data were presented as mean \pm standard deviation (SD). Analysis of variance (ANOVA) followed by Bonferroni's test was used to determine the significant differences between groups. Values of P less than 0.05 were considered significant.

Results

Western blot analysis to detect expression of truncated product

PCR products were cloned in pcDNAIneo vector (Figure 1.a). Presence of a variant was confirmed by digestion with restriction enzyme (Fig1.b). The variant clones were transfected into HeLa cell and the stable cell

lines were generated. The expression of the variant product was confirmed by RT-PCR (Figure 1.c) and western blot analysis (Figure 1.d). For further analyses,

Alkylating agent sensitivity detection by MTT assay

Cytotoxicity of Pol $\beta\Delta_{208-304}$ cells were determined and compared with the control HeLa cells and HeLa transfected with vector alone (data not shown) by the MTT assay. Cell cytotoxicity assay indicate that transfected cell are more sensitive to NMU and MMS than control cell line.

Treatment of Pol $\beta\Delta_{208-304}$ cells and control cells with MMS (1 μ M -750 μ M) results in the decrease of the percentage of cell survival. Treatment of this cell lines with 10 μ M MMS resulted in the 89% cell survival compared to 94% cell survival noticed in HeLa control cells. In 250 μ M doses of MMS, 41% control cells are dead when same concentration of MMS, resulted in 70 % of cell death in transfected cell line. These results showed that the Pol $\beta\Delta_{208-304}$ cells were more sensitive to MMS treatment than parental HeLa cell line (Figure 2.a). Similar trend were observed when Pol $\beta\Delta_{208-304}$ cells were treated with NMU (1-500 μ M) (Figure 2.a). The results show that almost 50% HeLa cells died after treatment with 250 μ M NMU (Figure 2.b) in control cell whereas 89% Pol $\beta\Delta_{208-304}$ transfected cells died at the same concentration of NMU. Treatment resulted in the death of 21%, 33%, 51%, and 75% of cell death at concentrations of 50 μ M, 100 μ M, and 250 μ M, 500uM respectively in HeLa cells where Pol $\beta\Delta_{208-304}$ exhibited 34%, 65%, 91%, 98% of cell death at the same concentrations of NMU. These data indicated that Pol $\beta\Delta_{208-304}$ could increase the sensitivity towards NMU and MMS at a significant level.

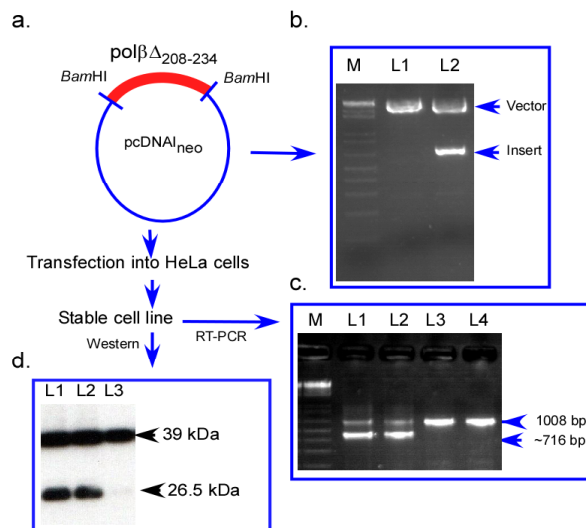


Figure 1. a Schematic Construction of recombinant plasmid inserted with Pol $\beta\Delta_{208-304}$. b Cloning of Pol $\beta\Delta_{208-304}$ in vector. M; Marker, L1; vector without insert, L2; vector with insert of 716bp. c RT-PCR product of Pol $\beta\Delta_{208-304}$ transfected cell line and normal cell line and vector alone. M; Marker, L1 and L2: RT product of Pol $\beta\Delta_{208-304}$ transfected cell line 1 and cell line ; L3: RT-PCR product of control cell line and L4: HeLa cell transfected with vector alone. d Detection of Expression of DNA pol β by western blot analysis. L1 and L2: Pol $\beta\Delta_{208-304}$ transfected cell line 1 and 2 respectively. L3: HeLa cell line without transfection

H₂O₂, UV sensitivity assay by MTT

Pol $\beta\Delta_{208-304}$ transfected cells are treated with oxidative stress producing agent H_2O_2 of different concentrations and radiating agent UV at different doses. When Pol $\beta\Delta_{208-304}$ cells were treated with H_2O_2 of different concentrations (0.01-1mM), almost 50% cell died in control cells after treatment with 0.5mM H_2O_2 for 6 hrs whereas cell death rate is much higher (66%) in Pol $\beta\Delta_{208-304}$ cell line.

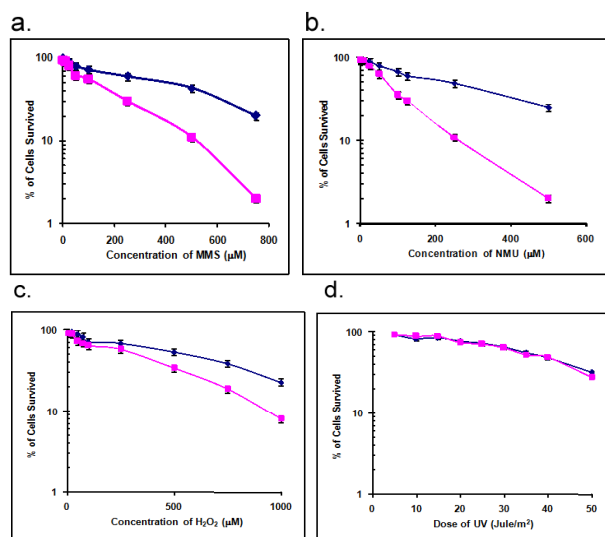


Figure 2. a & b Effects of Carcinogen on Cell Viability were Examined by Using the MTT (thiazolyl blue) assay. This assay monitors conversion of MTT to insoluble purple formazan in a reaction catalyzed by mitochondrial dehydrogenases in viable cells. -▲- denotes HeLa cells, whereas -■- indicates HeLaPol $\beta\Delta$ cells. a; Graphically represent Cell viability along with treatment of MMS of different concentration. b; Cell viability with different concentration of NMU. c; Cell viability with different concentration of H_2O_2 , d; Cell viability with different concentration of UV. The data represent mean \pm SD (n=3)

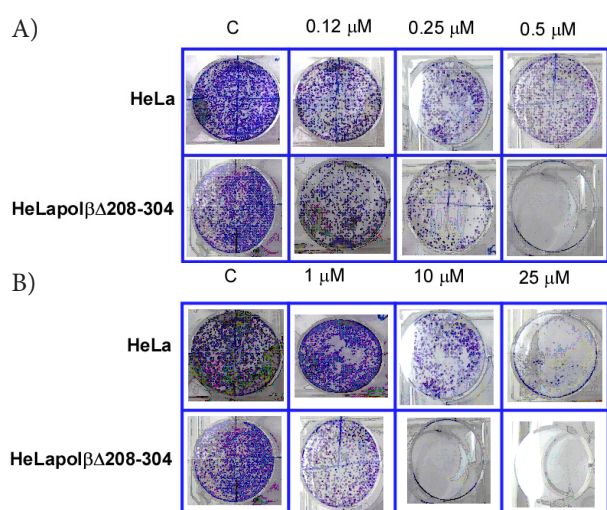


Figure 3. Colony Forming Assay by Treatment of MMS in HeLa Control (Upper) and Pol $\beta\Delta_{208-304}$ (Lower) Transfected Cell Line. Here C indicates-control cell line, and 0.12, 0.25, 0.5 μ M are concentration of MMS treated in cultured dishes. b. Colony forming assay by treatment with NMU C indicate Control cell line and 1 μ M, 10 μ M, 25 μ M were treated dish by different concentration of NMU respectively

Treatment resulted in the death of 6, 29, 80 % cells at concentrations of 10 μ M, 100 μ M and 750 μ M whereas the Pol $\beta\Delta_{208-304}$ exhibited 9, 36, 84 % cell death at the same concentrations of H_2O_2 (Figure 2.c)

Treatment of Pol $\beta\Delta_{208-304}$ cells with UV of 5-50 J/m² resulted same sensitivity for both control cells and Pol $\beta\Delta_{208-304}$ transfected cells. Treatment of this cell line with UV resulted 94%, 89%, 84%, 74%, 71% cell survival, where in HeLa cell 93%, 83%, 85%, 77%, 73% cells are survived in 5-25 J/m² irradiation indicate that sensitivity are almost same. (Figure 2d)

Colony forming assay by treating with alkylating agent (MMS, NMU), H_2O_2 , UV

Results of colony forming assay indicate that transfected cells are more sensitive to MMS, NMU, H_2O_2 than HeLa cell line or HeLa cells transfected with vector DNA alone (Figure 3 a & b and Figure 4 a,b,c,d). On the other hand, UV does not affect the transfected cells when compared with HeLa alone (Figure 4 b).

Study of apoptosis

Sensitivity study by DNA fragmentation assay against MMS, NMU, H_2O_2 , and UV: After treatment with toxic agent, DNA fragmentation was detected as small bands in a laddering pattern in 1.8% agarose gel. The results show that in NMU (100 μ M) and MMS (250 μ M) treatment the DNA fragmentation is prominent in 6 hrs in Pol $\beta\Delta_{208-304}$ cells whereas the fragmentation was detected in 9 hrs in treated HeLa cells. In higher doses of chemical treatment transfected cells are showing more degradation of DNA.

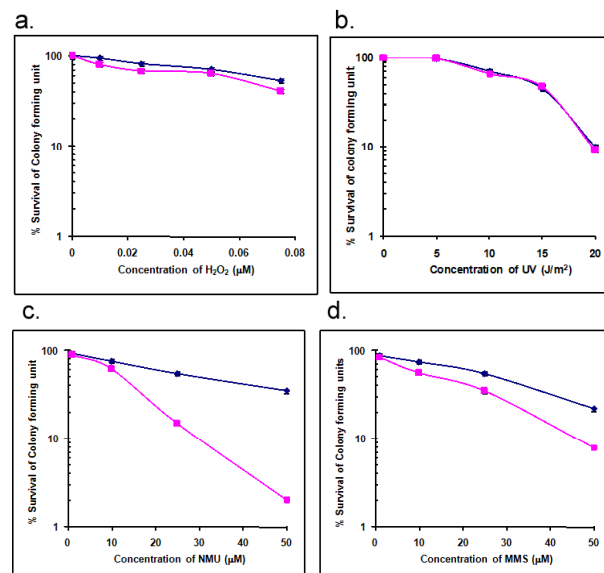


Figure 4. a & b Graphical representation of colony forming assay. Effect of H_2O_2 and UV on the colony forming assay of HeLa and HeLa-pol $\beta\Delta$ cell. -▲- denotes HeLa cells, whereas -■- indicates HeLaPol $\beta\Delta$ cells. a. Shows percentage of colony formation after treatment with different concentrations of H_2O_2 b. Graphical presentation of percentage of colony formation after treatment with different doses of UV. c & d Effect of MMS and NMU on the colony forming assay of HeLa pol $\beta\Delta$. c Shows percentage of survived colony forming units after treatment with different concentrations of NMU. d. Graphical presentation of percentage of survived colony forming units after treatment with different doses of MMS. The data represent mean \pm SD (n=3).

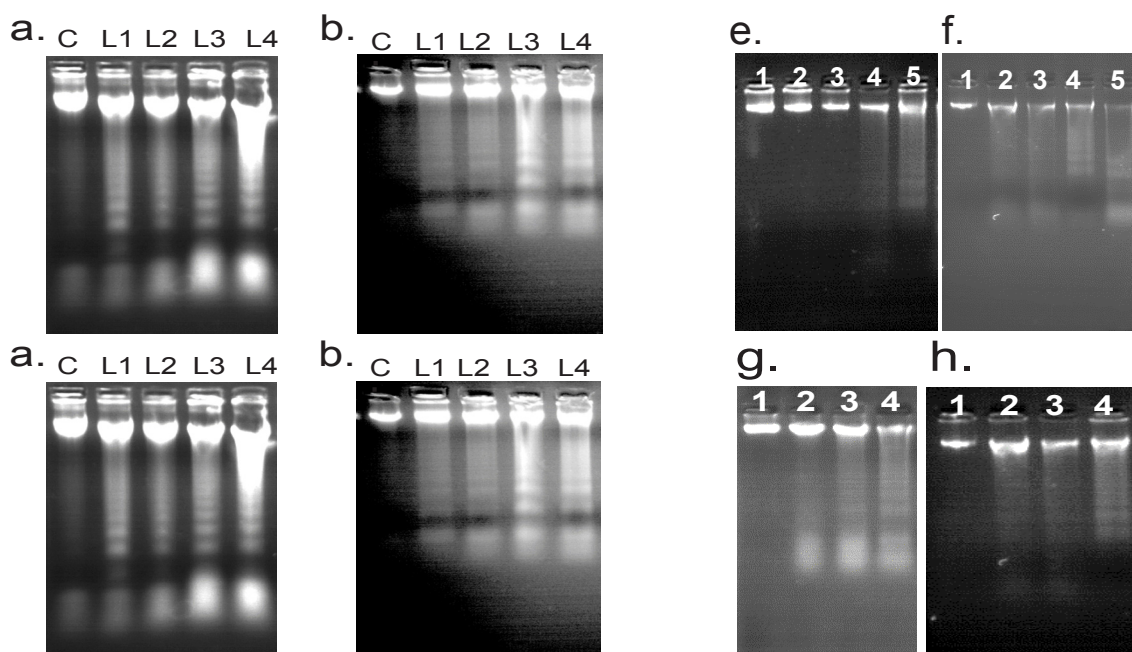


Figure 5. a&b: Study of Apoptosis by DNA Fragmentation after Treating with UV(10 J/m²). a; DNA laddering after treatment with UV in HeLa cell line b; DNA laddering after treatment with UV in pol $\beta\Delta 208-304$ cell line respectively. In this figure, C indicates control, L1 indicate UV treatment after 6 hrs, L2 indicate UV treatment after 9 hrs, L3 indicate UV treatment after 12hrs, L4 indicate UV treatment after 24hrs. c & d: Study of apoptosis by DNA laddering after treatment with NMU (100 μ M). c; the DNA laddering after treatment with doses of NMU in HeLa cell line d; DNA laddering after treatment with NMU in pol $\beta\Delta 208-304$ cell line respectively. C indicate Control, L1 indicate NMU treatment after 6 hrs, L2 indicate NMU treatment after 9hrs, L3 indicate NMU treatment after 12hrs, L4 indicate NMU treatment after 24hrs. e & f: DNA laddering showing after treatment with MMS (250 μ M). e; the DNA laddering after treatment with doses of MMS in HeLa cell line f; DNA laddering after treatment with MMS in pol $\beta\Delta 208-304$ cell line respectively. 1 indicates control; 2 indicate MMS treatment after 3 hrs; 3 indicate MMS treatment after 6hrs; 4 indicate MMS treatment after 9hrs; 5 indicate MMS treatment after 12hrs. g & h Study of apoptosis by DNA fragmentation assay g; the DNA laddering after treatment with different doses of H₂O₂ in HeLa cell line h; DNA laddering after treatment with H₂O₂ in pol $\beta\Delta 208-304$ cell line respectively. C indicate control, L1 indicate treatment of 25 μ M H₂O₂, L2 indicate 50 μ M H₂O₂, L3 indicate 100 μ M H₂O₂

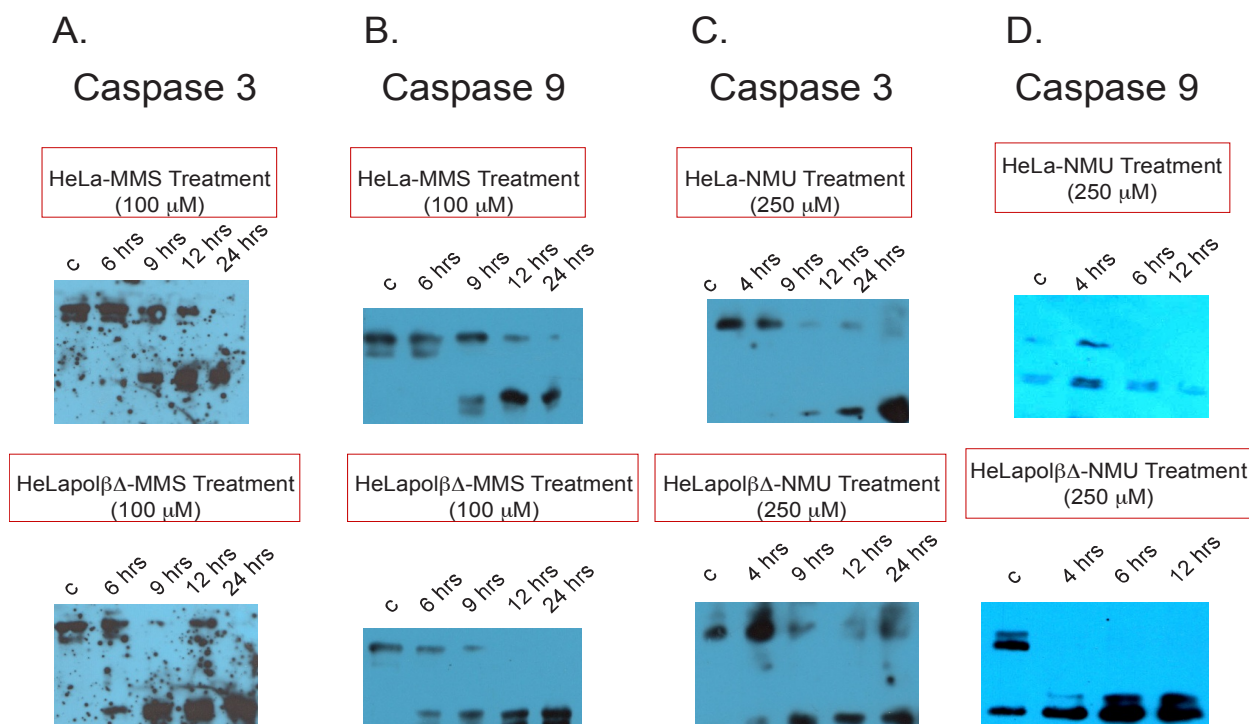


Figure 6. Study of Apoptosis by Caspase assay Detected by Western blot Analysis in HeLa Cell Line and Pol $\beta\Delta 208-304$ Cell Line of Different Time Period. Here C indicate cell line of without treatment, 6hrs, 9hrs, 12hrs indicate cell line with treatment after 6hrs, 9hrs, 12hrs respectively. A: caspase 3 activation by treatment with MMS (100 μ M). B: Caspase 9 activation by MMS (100 μ M) treatment. C: Caspase 3 activation by NMU (250 μ M) treatment. D: Caspase 9 activation by NMU (250 μ M) treatment

Caspase 3

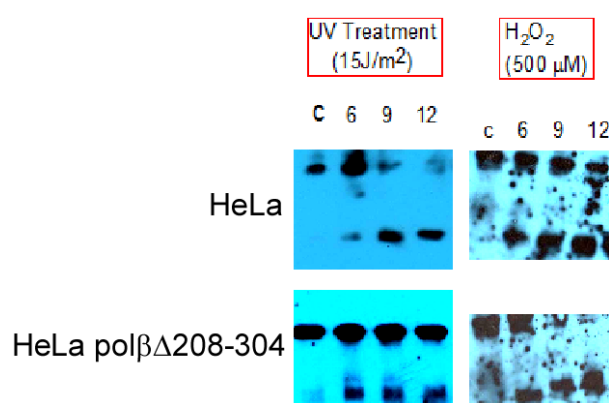


Figure 7. Caspase 3 Activation Detection by UV and H_2O_2 Treatment. C indicate control cell line 6,9,12 indicate 6hrs, 9hrs, 12hrs respectively of UV ($15J/m^2$) and H_2O_2 treatment

Cells are more sensitive to NMU than MMS (Figure 5 c, d, e, f). In case of UV treatment, at the dose of $10 J/m^2$, the DNA laddering was prominent in $Pol\beta\Delta_{208-304}$ cells (Figure 5 a, b) as well as in HeLa control indicate that $pol\beta\Delta$ transfected cells are equally sensitive to UV like control cells. DNA fragmentation assay indicate that $Pol\beta\Delta_{208-304}$ cells are more sensitive to NMU and MMS than UV. HeLa and $Pol\beta\Delta_{208-304}$ cells were treated with different concentrations of $25\mu M$ - $100\mu M$ of H_2O_2 indicate that $pol\beta\Delta$ are little more sensitive to H_2O_2 (Figure 5 g & h).

Caspase activation detection

To gain further insight into whether caspases are involved in alkylating agent mediated apoptotic signals, we determined the activation of caspase-9 and -3 in HeLa and $Pol\beta\Delta_{208-304}$ cells. Binding of cytochrome C to Apaf-1 results in the cleavage of procaspase-9, which in turn activates caspase-3. In order to determine whether caspases are activated we measured the changes in caspases-9 and -3 activity in HeLa and $Pol\beta\Delta_{208-304}$ cells after treatment.

In this study we noticed that after 6 hrs of treatment with NMU and MMS treatment $Pol\beta\Delta_{208-304}$ transfected cell activation started where no activation is observed in control cell line (Figure 6 A, B, C, D). In the present study we also noticed UV and H_2O_2 induced activation of caspase-3 in both HeLa and $pol\beta\Delta$ cells, but the activation rate is same in $Pol\beta\Delta_{208-304}$ cells compared to HeLa cells (Figure 7). As shown in Figure 7, the activation of caspase 3 occurred at the dose of $15 J/m^2$ in HeLa cell, whereas the same activation was detected in $Pol\beta\Delta_{208-304}$ cells at same dose.

Caspase 3 are activated at 6 hrs of UV treatment in case of HeLa control and $Pol\beta\Delta_{208-304}$ cell line indicate this cell line is sensitive to UV treatment but not due to transfection of $pol\beta\Delta$, the activation occurs as early as 6 hrs. Therefore this result suggests that $Pol\beta\Delta_{208-304}$ are not sensitive to UV treatment. In case of H_2O_2 after 6hrs of ($500\mu M H_2O_2$) treatment caspase activation occurs in control and transfected cell line.

Discussion

$Pol\beta\Delta_{208-304}$ variant has large deletion of 97 amino acids in the catalytic part of amino acids residues 208 to 304. The variant of 716 bp has deletion in Exon 11-13. The exon 11 has nucleotidyl selection activity; exons 12 and 13 have dNTPs selection activity (Idris et al., 2006). Hence deletion of this part may affect polymerizing activity but single strand binding and double strand binding activity presumed to be remained same that have nucleotidyl transferase activity and dNTPs selection activity. In this variant of DNA $pol\beta$ one of the three aspartates at position 256 (Asp190, Asp192, and Asp256) necessary for catalysis reaction is missing which is located at exon 12 [24-26] and Amino acids Tyr271, Phe272, Asn 279 and Arg283 located in catalytic domain that have role in minor groove interaction thus maintaining the fidelity of the polymerization are also missing (Dong et al., 2002; Sweasy et al., 2005; Lang et al., 2007). The fidelity of polymerization will also be affected due to deletion of Exon 13. Bhattacharyya and Banerjee (1997) have shown that 87 bp deletion of $pol\beta$ in breast, colon, and lung cancer has a dominant negative (DN) effect (Bhattacharyya and Banerjee 1997). As the alteration is heterozygous, this variant will compete with WT $pol\beta$ for DNA binding, thus may act as dominant negative manner. The hindrance of the function of wild-type $pol\beta$ may sensitize the cancer cells to the alkylating agent and thus may the target of an effective therapy.

The Wild type (WT) $pol\beta$ consists of 335 amino acid residues. The variant form has a deletion of 97 amino acids in catalytic part. The sensitivity of this new cell line, $Pol\beta\Delta_{208-304}$, was tested by MTT assay after treating cells with alkylating agents and oxidative stress generating agent UV, H_2O_2 . $Pol\beta\Delta_{208-304}$ cells exhibit greater sensitivity to alkylating agent and less sensitivity towards H_2O_2 . $Pol\beta\Delta_{208-304}$ has deletion of exon 11-13. $Pol\beta$ -DNA complex binds to the incoming nucleoside triphosphate (dNTP) and place it within the active site. Subsequently, conformational changes occur, the incorrect dNTP binding results in a poor fit, and disrupt DNA repair mechanism. As this newly identified mutant lost dNTPs selection region it may incorporate wrong bases that may results poor fitting or no repair.

It has been shown that Mouse embryonic fibroblast cells deficient in $pol\beta$ are highly sensitive to alkylating agents. The lesions produced by these agents are repaired by BER, thus confirming the role of $pol\beta$ in this repair pathway (Sobol et al., 1996; Ochs et al., 1999). If this variant is a dominant negative one, then it is thought to bind to glycosylase and AP endonuclease treated DNA sites hence may block access of wild type $pol\beta$. If this hypothesis is true, then we may expect an increased sensitivity of $pol\beta\Delta_{208-304}$ variant expressing HeLa cells to alkylating drugs. Significant increase in alkylating drug sensitivity was observed in the $pol\beta\Delta$ cells suggesting inhibition of BER (Figure 2,3,4).

The MTT assay results have shown that the $pol\beta\Delta_{208-304}$ cells are more sensitive to MMS as well as NMU (Figure 2.a & b). In this study, the MMS, NMU treatment

of pol $\beta\Delta_{208-304}$ cells have showed enhanced DNA fragmentation than that of HeLa cells. DNA laddering of pol $\beta\Delta_{208-304}$ cells was apparent at lower doses of MMS, NMU (Figure 5).

If we assume that pol $\beta\Delta_{208-304}$ is a dominant negative mutant, then these cells are not expected to be more sensitive to UV irradiation. The nucleotide excision repair (NER) pathway repairs DNA damage resulting from UVC irradiation which produces primarily pyrimidine dimers. Pol β is not involved in this process. No significant difference in UVC sensitivity could be observed between HeLa cells and HeLa pol $\beta\Delta_{208-304}$ cells (Figure 2d, 4b). This data also indicates that the presumed dominant negative mutant of pol β does not interfere with NER.

Like ionizing radiation, causes oxidation at abasic sites, purines and pyrimidines, and SSBs occurs by H₂O₂ treatment (Li et al., 1997). H₂O₂-treated pol β null and normal cells show no difference between repair of SSBs, indicating pol β independent repair of DNA damage (Peus et al., 1999). The oxidized AP sites may not be effectively excised by pol β after H₂O₂ treatment (Pelle et al., 1990). In our study, pol $\beta\Delta_{208-304}$ cells are showing same sensitivity as that of the HeLa control cell line. Therefore, the data also indicates that radiation and H₂O₂ induced damage is being repaired by pol β -independent long patch repair pathway.

As this variant has its 8-kDa ss DNA binding domain is intact, it will bind to the SSBs and the 5'-deoxyribose phosphate will be removed by the dRP lyase activity of pol β . But as the catalytic domain is not intact, the sealing of the abasic site may not be possible. Therefore SSBs will be accumulated that may trigger of cell death particularly apoptosis. Therefore, we measured the mitochondrial damage related caspases such as caspase 9 and caspase 3. In addition, as the activation of caspase 9 and caspase 3 activity leads to PARP degradation, we have compared the PARP degradation pattern in these two cell lines. The first step for the reactive oxygen species (ROS) mediated apoptosis is that cytochrome c binds to Apaf thus forming a complex called apoptosome which recruits and activates procaspase 9. Then caspase 9 cleaves inactive procaspase 3 to form active caspase 3. We thus examined the effects of MMS, NMU, H₂O₂ and UV on the processing of caspase 9 and caspase 3, which are directly activated by caspase 9 (Hussain et al., 1995; Lawley et al., 2000). The results of caspase assay also indicate more activation of caspases in pol $\beta\Delta_{208-304}$ transfected cell line.

In conclusion, we have identified a variant form of pol β , which is specific for ovarian cancer, has a potential to be a dominant negative mutant. This presumed to be pol β DN mutant, has intact N-terminal DNA binding domain may be disrupting the function of the WT pol β by binding to the specific damaged DNA (AP sites). As this DN mutant lost its catalytic domain partially thereby lost the catalytic ability. In addition, when this DN mutant binds to the gap it may also hinder the binding of other DNA polymerases. Hence it may inhibit both SP BER and LP BER. Similar observation was made (Clairmont and Sweasy, 1996; Clairmont et al., 1998) where a pol β mutant containing yeast cells are sensitive to alkylating agent but not to UV indicating an involvement in BER but

not in NER. Another study by Bhattacharyya and Banerjee showed an heterozygous variant form without catalytic domain exhibited substantially decreased BER activity and increased sensitivity to MNNG (Bhattacharyya and Banerjee, 1997). The alkylated DNA is not exclusively repaired by the BER pathway but mismatch repair is also involved in the removal of damage resulting from alkylating agents (deWind et al., 1999). But in this case the pol $\beta\Delta_{208-304}$ construct also blocks this escape route.

The activation of caspase 9 became apparent and progressed at least up to 6 hours (Figure 7) after alkylating agent treatment in pol $\beta\Delta_{208-304}$ than control cell line. pol $\beta\Delta_{208-304}$ transfected cells susceptible to chemotherapeutic agent like alkylating agent. This would represent a clinical significance if translated into clinical setting because resistance to radiation or chemo during the relapse of the disease could be potentially overcome by this approach. Hence hopefully in the near future, strategies based on RNAi will be ready for preclinical or clinical trials.

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